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13. ABSTRACT *(Maximum 200 Words)*

Neurofibromatosis type 1 predisposes to the growth of both benign and malignant tumors. Genetic factors, in addition to inactivation of the NF1 gene itself, clearly play a role in tumor development. Our research is focused on identifying specific DNA sequences and genetic mechanisms important in the development of cutaneous neurofibromas. We are analyzing NF1 microdeletions, which are associated with an early onset, and subsequent heavy burden, of cutaneous neurofibromas. We identified recombination hotspots where breakpoints in ~75% of patients with NF1 microdeletions occur and developed assays that detect microdeletions in a patient blood sample. Near the recombination hotspots where microdeletions occur, we identified unique sequence elements that may mediate recombination at these sites and make some chromosomes susceptible to NF1 microdeletion. Unexpectedly, we found that NF1 microdeletions that arise in a somatic cell during early embryogenesis occur at different sites. An understanding of how NF1 microdeletions occur, whether some individuals are more susceptible, and why they potentiate the development of neurofibromas is important for patient care, genetic counseling, and the design of effective pharmacological intervention strategies.

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Introduction

Neurofibromatosis type 1 affects 1/4000 individuals worldwide and predisposes to the growth of both benign and malignant tumors. Previously, we hypothesized that the early age at onset of cutaneous neurofibromas observed in patients with NF1 microdeletions was caused by the co-deletion of a second locus that potentiated neurofibromagenesis (1, 2, 3). This hypothesis is supported by our recent data demonstrating that NF1 microdeletion breakpoints are clustered at repetitive elements (NF1-REPs) that flank the NF1 locus (4). In this application we propose to test the following hypotheses. [1] NF1 microdeletion breakpoints occur at a small segment that defines a meiotic recombination hotspot(s) within the 15-100kb NF1-REP element and that homologous recombination at the hotspot is facilitated by a nearby recombinogenic element. [2] Polymorphism in NF1-REP number, orientation, and/or complexity predisposes certain individuals to NF1 microdeletion and the consequent high neurofibroma burden. [3] NF1 microdeletion increases the risk of developing a solid tumor malignancy. [4] NF1-REP-mediated NF1 microdeletion in somatic cells is an underlying mechanism of loss of heterozygosity at the NF1 locus in malignant tumors of NF1 patients. This research will identify specific genetic loci and mechanisms that play a role in tumor development in NF1 patients.

Body

Original Statement of Work

Year 1:

- **Development of a probe to detect NF1-REP mediated deletion junction fragments**

To date, we have succeeded in developing five assays to detect NF1 microdeletion junction fragments in about 75% of patients. Previously, we had shown that the majority of 1.5 Mb NF1 microdeletions occur by recombination between flanking repetitive elements [termed NF1-REP-P (proximal) and –M (medial)] of about 60 kb in length (4). Initially it was not clear exactly how a probe could be developed to detect NF1-REP mediated deletion junction fragments because we did not know whether the deletion breakpoints were clustered in or scattered throughout the NF1REP elements. This year we mapped NF1 deletion breakpoints to the sequence level and discovered that 46% of patients with entire gene deletions (N=54) have breakpoints that map to a 2 kb recombination hotspot within the NF1-REPs that we designated PRS1 (Paralogous Recombination Site 1) ((5); see appendix). A probe to detect deletions at PRS1 was developed by identifying rare nucleotide differences between NF1-REP-P and NF1-REP-M. These sites were used to design primers that would specifically amplify from each REP. A 3.4 kb amplicon is produced from deleted chromosomes with breakpoints at PRS1; however, amplification of the 1.5 Mb segment from normal chromosomes cannot occur.

Using similar strategies, we developed assays that detect four other NF1-REP mediated deletion junction fragments. These assays detect microdeletions at 1) new breakpoints adjacent to PRS1 found in about 6% of microdeletion patients (N=54). 2) a second recombination hotspot, termed PRS2, located 20 kb distal to PRS1in the NF1REP-P and –M elements. This assay demonstrated that about 30% of microdeletion patients (N=54) have breakpoints at PRS2. 3) a unique breakpoint in a novel location in the NF1-REPs. The prevalence of breakpoints at this site is as yet undetermined. 4) a unique breakpoint in a duplicated gene that lies outside of the NF1-REP elements. In contrast to all the other

microdeletions, which occurred in the germline, this one occurred in the somatic tissue in a patient who is an NF1 microdeletion mosaic. The importance of this microdeletion is discussed below.

- **Sequence and structural analysis of NF1REP-P and –M**

We have identified unique sequence elements near the recombination hotspots PRS1 and PRS2 that may mediate recombination at these sites. Two types of GA rich elements have been identified. The first is a dinucleotide repeat, which is invariant in NF1REP-P and highly polymorphic in NF1REP-M. The second is set of 3 pairs of GA rich motifs that are regularly spaced throughout the PRS1 and PRS2 regions. These may be involved in stem-loop formations and or chromatin structures that are favorable to recombination. We are currently investigating how this element may influence homolog pairing and recombination. The variant dinucleotide repeat is of particular interest because chromosomes with specific variants may predispose to recombination and microdeletion. This hypothesis is currently being tested by examination of ancestral chromosomes 17 that underwent de novo microdeletion.

We have determined that mitotic recombination events that lead to NF1 microdeletion occur at different sites than the germline recombination events. Patients who are mosaic for an NF1 microdeletion do not have breakpoints at PRS1 or PRS2. This implies that mitotic microdeletions occur by a different mechanism at a novel site(s). This is important to determine since the somatic NF1 loss that occurs during tumorigenesis may occur by a similar mechanism. We have mapped one mitotic NF1 microdeletion breakpoint to the sequence level, developed a deletion junction fragment specific assay, and are using that to determine if other patients mosaic for NF1 microdeletions have breakpoints clustered at this location.

We have identified another partial NF1-REP (designated P2) that is located just proximal to the NF1 gene. Homologous recombination between NF1-REP-P2 and NF1-REP-M (located distal to NF1) resulted in an NF1 microdeletion in one patient. NF1-REP-P2 has limited homology to NF1-REP-M, which makes this rearrangement mechanistically important to investigate.

In order to further understand NF1-REP structure and how these elements became inserted near the NF1 gene, we constructed a physical map of the mouse *nf1* microdeletion region. The mouse genome does not have NF1-REP elements, but the arrangement of genes in the region provides information regarding the ancestral chromosome into which NF1-REPs were inserted. We found a high degree of conservation of the NF1 linkage group in mouse. Although NF1-REPs and other small duplicons are absent, the order of orthologs of known ESTs/genes in the microdeletion region are conserved. This implies that the insertion of NF1-REPs was not accompanied by gross rearrangement of the chromosome.

Years 2 and 3:

- **Ascertainment and sample collection of NF1 patients with tumors**

  Work will begin this year.
- **Employ the automated NF1 gene dosage assay to determine if the frequency of NF1 microdeletions is greater in patients that develop malignancies**

  Work will begin this year.

Year 3:

- **Genotype grandparents to determine meiotic mechanism of microdeletion**

  Work will begin this year.
- **Employ assay to detect NF1REP-mediated deletion junction fragment in tumor tissue of NF1 patients**

Using the PRS1 and PRS2 deletion junction-specific amplification assays, we have screened over 30 tumors and have not detected NF1 microdeletions at these sites. This is consistent with our hypothesis that mitotic NF1 microdeletions occur at novel sites. This year, tumor DNAs will be screened to determine if they harbor NF1 microdeletions with breakpoints in the novel region identified in NF1 microdeletion mosaic patients.

Key Research Accomplishments

- We identified two recombination hotspots where ~75% of germline NF1 microdeletions occur. This is important because 1) these hotspots can be analyzed in detail to investigate why recombination is favored at these sites and 2) the majority of microdeletion patients will have virtually the same genotype and will therefore constitute an important patient cohort for genotype/phenotype analyses.
- We developed simple and reliable assays that detect the presence of a germline NF1 microdeletion in a patient blood sample. This will facilitate identification of patients with microdeletions.
- We found that NF1 microdeletions that arise during mitosis in embryonic development occur at different sites than germline microdeletions. This implies that mitotic NF1 microdeletions occur by a novel mechanism; a mechanism that may also occur during tumorigenesis.
- We sequenced the breakpoint and developed an amplification assay for one case with a mitotic NF1 microdeletion. Such cases are mosaic for an NF1 microdeletion, their prevalence in the patient population is unknown, but has important counseling and reproductive implications.
- We identified unique sequence elements near the recombination hotspots PRS1 and PRS2 that may mediate recombination at these sites.
- We identified a polymorphic DNA microsatellite in NF1REP-M, which is invariant in NF1-REP-P, near the recombination hotspots. This may make some chromosomes susceptible to NF1 microdeletion.
- We found a high degree of conservation of the NF1 linkage group in mouse. Although NF1-REPs and other small duplicons are absent, the order of orthologs of known ESTs/genes in the microdeletion region are conserved. This implies that the insertion of NF1-REPs in the ancestral primate genome was not accompanied by gross rearrangement.

Reportable Outcomes

- Manuscripts
  - López-Correa C, Dorschner MO, Brems H, Lázaro C, Clementi M, Upadhayaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Stephens K, Legius E. 2001. Recombination hotspot in NF1 microdeletion patients. Hum Mol Genet 10:1387-1392.
  - Dorschner MO, López-Correa C, Brems H, Lázaro C, Clementi M, Upadhayaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Legius E, Stephens, K. A second paralogous recombination hotspot in NF1 microdeletion patients. In preparation.

- Invited presentations
  - Stephens K. Mechanisms of Contiguous Gene Syndromes. Continuing Medical Education Series: The Twentieth Century and the Impact of Technology on Clinical Genetics. Seattle, April 21, 2001.
  - Stephens K. Evidence for a tumor-modifying gene in NF1. National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. Aspen, CO, June, 2001.
  - Stephens K. "Paralogous recombination: mechanism of chromosomal del / dup syndromes", University of Utah, Department of Pediatrics. June 18, 2001.
- Conference talks:
  - Stephens K. Evidence for a tumor-modifying gene in neurofibromatosis type 1. Ninth European Neurofibromatosis meeting, April 6-8, 2001, Venice, Italy.
- Abstracts
  - Stephens K, Dorschner MO, Leppig KA, Sybert VP. Evidence for a tumor-modifying gene in neurofibromatosis type 1. Ninth European Neurofibromatosis meeting, April 6-8, 2001, Venice, Italy.
- Development of diagnostic assays
  - Simple and reliable assays were developed to detect the presence of an NF1 microdeletion in a patient blood sample.
- Training
  - Postdoctoral fellow Michael O. Dorschner was trained in the molecular genetics of neurofibromatosis type 1.
  - Postdoctoral fellow Stephen H. Forbes is currently being trained in the molecular genetics of neurofibromatosis type 1.
- Funding Applied for based on work supported by this award
  - Title: Prognosis of cognitively impaired children with NF1 as adults.
    Principal Investigator: Elizabeth Ochoa, MD. Co-Investigator: Karen G. Stephens, PhD
    Submitted July, 2001 to U.S. Army Medical Research and Material Command
  - Title: Identifying a gene that potentiates neurofibromagenesis.
    Principal Investigator: Karen G. Stephens, PhD
    Submitted July, 2001 to U.S. Army Medical Research and Material Command

Conclusions

Neurofibromatosis type 1 affects 1/4000 individuals worldwide and predisposes to the growth of both benign and malignant tumors. Genetic factors, in addition to defects in the NF1 gene itself, clearly play a role in tumor development. Our research is focused on identifying specific DNA sequences and genetic mechanisms important in the development of cutaneous neurofibromas, which occur in virtually all NF1 patients, and in the development of solid malignancies, which occur in about 5% of NF1 patients. We have been analyzing NF1 microdeletions that are associated with an early onset, and subsequent heavy burden, of cutaneous neurofibromas. We identified recombination hotspots where ~75% of NF1 microdeletions occur and developed assays to detect microdeletions in a patient blood sample. This is important because 1) these hotspots can be analyzed in detail to investigate why recombination is favored at these sites and 2) the majority of microdeletion patients will have virtually the same genotype and will therefore constitute an important patient cohort for genotype/phenotype analyses. Near the

recombination hotspots where microdeletions occur, we identified unique sequence elements that may mediate recombination at these sites and make some chromosome susceptible to NF1 microdeletion. In addition, we found that the NF1 microdeletions in some patients occurred early during embryonic development. These microdeletions arose by mitotic recombination events, which appear to occur by a different mechanism than germline microdeletion events. An understanding of how NF1 microdeletions occur, whether some individuals are more susceptible, and why they potentiate the development of neurofibromas is important for patient care, genetic counseling, and the design of effective pharmacological intervention strategies.

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5. C. Lopez-Correa, et al., *Hum Mol Genet* **10**, 1387-1392. (2001).

Appendices

Manuscript: López-Correa C, Dorschner MO, Brems H, Lázaro C, Clementi M, Upadhayaya M, Dooijes D, Moog U, Kehrer-Sawatzki H, Rutkowski JL, Fryns J-P, Marynen P, Stephens K, Legius E. 2001. Recombination hotspot in NF1 microdeletion patients. Hum Mol Genet 10:1387-1392.

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Recombination hotspot in *NF1* microdeletion patients

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Neurofibromatosis type 1 (NF1) patients that are heterozygous for an *NF1* microdeletion are remarkable for an early age at onset and an excessive burden of dermal neurofibromas. Microdeletions are predominantly maternal in origin and arise by unequal crossover between misaligned NF1REP paralogous sequence blocks which flank the *NF1* gene. We mapped and sequenced the breakpoints in several patients and designed primers within each paralog to specifically amplify a 3.4 kb deletion junction fragment. This assay amplified a deletion junction fragment from 25 of the 54 unrelated *NF1* microdeletion patients screened. Sequence analysis demonstrated that each of the 25 recombination events occurred in a discrete 2 kb recombination hotspot within each of the flanking NF1REPs. Two recombination events were accompanied by apparent gene conversion. A search for recombination-prone motifs revealed a χ-like sequence; however, it is unknown whether this element stimulates recombination to occur at the hotspot. The deletion-junction assay will facilitate the prospective identification of patients with *NF1* microdeletion at this hotspot for genotype–phenotype correlation studies and diagnostic evaluation.

INTRODUCTION

Haploinsufficiency for neurofibromin, the protein product of the *NF1* gene, causes the autosomal dominant disorder neurofibromatosis type 1 (NF1) (reviewed in refs 1–3). While the majority of cases are caused by subtle private mutations which predict truncation of neurofibromin (4,5), an estimated 5–22% are heterozygous for a germline deletion spanning the 350 kb *NF1* locus (6–9). Early reports that deletion patients were remarkable for facial anomalies and an early age at onset of cutaneous neurofibromas, or for excessive numbers relative to age in cases for which age at onset was unknown (10,11), have been confirmed by the identification of additional patients (7,9,12–16). A few deletion cases without this phenotype have been reported (7,9,16), but because the extent of the deletions was not delineated, it is unclear whether they involved the same loci. These observations led to the hypothesis that the *NF1* microdeletion resulted in haploinsufficiency for neurofibromin and for the product of a second contiguous locus, which together potentiated neurofibromagenesis (11,17).

This hypothesis was supported by recent data showing that 80% (n = 17) of microdeletion breakpoints were clustered at paralogous sequences which flank the *NF1* gene (17). These paralogs, termed NF1REP-P and -M for proximal and medial, respectively, are ~85 kb in length and in direct orientation. NF1REP-mediated deletion most likely occurs by either interchromosomal recombination between misaligned NF1REP elements or intrachromosomal looping-out (17). The analysis of flanking polymorphic loci in family members of affected individuals with *de novo* microdeletions revealed that unequal crossing over during maternal meiosis I occurred in five out of six cases (18). This is consistent with earlier findings that ~80% of *NF1* microdeletions are maternal in origin (6,19).

Other than one expressed pseudogene and four expressed sequence tags (ESTs) (17), nothing is known of putative genes or sequence motifs in the NF1REP elements. Towards elucidating the molecular basis of *NF1* microdeletion and the genes involved, we mapped breakpoints, developed a deletion junction PCR assay, and analyzed the sequences of junction fragments. These analyses identified a hotspot for recombination between the NF1REP-P and -M paralogs.

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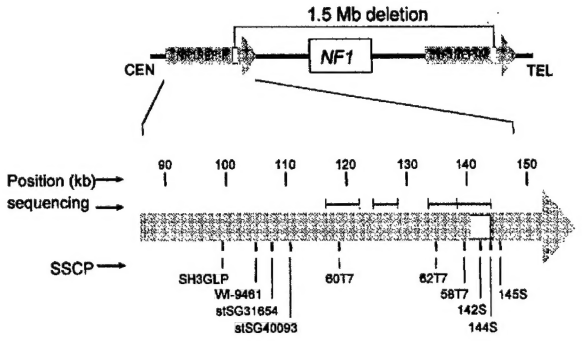

Figure 1. Localization of the *NF1* microdeletion breakpoints. The schematic shows the *NF1* gene and flanking paralogous elements NF1REP-P and -M in direct orientation. NF1REPs and the deleted region are not drawn to scale. *NF1* microdeletion occurs by homologous recombination between these NF1REP elements. The recombination hotspot within each NR1REP is depicted by white boxes. Below, the large gray arrow depicts the generic structure of both NF1REP elements with the position (kb) within the NF1REP shown relative to the sequence of BAC 271K11 (AC005562). PCR-amplified segments that were sequenced are shown above and the 10 loci analyzed by SSCP below.

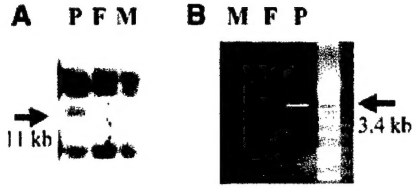

Figure 2. Detection of the *NF1* deletion junction fragment. (**A**) Southern blot of *Bcl*I-digested DNA from microdeletion patient 98-1 and his/her healthy parents was probed with a 200 bp PCR product from the breakpoint region of NF1REP-P (AC005562, nucleotides 143567–143766). The arrow indicates the novel 11 kb junction fragment present only in the patient. (**B**) The deletion junction PCR assay detects a novel 3.4 kb fragment in patient 98-1 but not in his/her healthy parents. F, father; M, mother; P, patient.

RESULTS

Refinement of *NF1* deletion breakpoint intervals

Our previous analyses of somatic cell hybrid lines carrying the deleted chromosome of 15 patients with 1.5 Mb deletions showed that in each case the *SH3GLP2* locus in NF1REP-P was retained, while *SH3GLP1* in NF1REP-M was lost (17, and patient C12 in this paper) (Fig. 1). Further refinement of the homologous recombination sites required identifying NF1REP-specific nucleotides. The strategy was to use the known NF1REP-P sequence to design primers to amplify sequences from a somatic cell hybrid line carrying a patient's deleted chromosome 17. Products were analyzed either by direct sequencing or single-stranded conformation polymorphism (SSCP) banding patterns. Results were compared with the sequence or banding pattern of NF1REP-P [AC005562, bacterial artificial chromosome (BAC) 271K11] and the draft sequence of NF1REP-M (AC023278, BAC 640N20; AC021852, BAC 474K4). As summarized in Figure 1, these results identified a common breakpoint interval of ~3 kb in seven of the 15 cases in which somatic cell hybrids were analyzed. As predicted, Southern blot analysis of *Bcl*I-digested DNA probed with a 200 bp fragment identified a novel deletion junction fragment of ~11 kb in a patient, but not in the patient's healthy parents (Figure 2A). This novel fragment was also detected in three additional unrelated *de novo* microdeletion patients, but not in their healthy parents (data not shown). Some of the breakpoints of the remaining eight hybrid lines carrying deleted chromosomes appear to cluster at a distinct site, but further sequence analysis is required for precise localization.

Detection of a deletion-specific junction fragment by PCR

To assay this recombination site in other *NF1* microdeletion patients, a deletion junction-specific PCR assay was developed. A forward primer specific for NF1REP-P and a reverse primer specific for NF1REP-M amplified a 3.4 kb junction fragment from DNA of patient C12 and from a somatic cell hybrid line carrying the deleted chromosome of this patient. Specificity of the primers was tested using P1-derived artificial chromosomes (PACs) from the different REPs (NF1REP-M, NF1REP-P, NF1REP-D, PAC with paralogous sequence from chromosome 19p), somatic cell hybrids with a deleted chromosome 17 and control DNA. This assay was then performed on DNA from 54 patients known to carry microdeletions extending beyond the borders of the *NF1* gene. The 3.4 kb deletion junction fragment was detected in 25 of 54 patients, but not in DNA from 75 control subjects. Figure 2B shows an example of this deletion junction PCR in patient 98-1 and his healthy parents. These results document the specificity of the assay in detecting only chimeric NF1REP sequences that arose from this specific deletion event. A chimeric NF1REP consisting of NF1REP-M and NF1REP-D could be excluded because this would not result in an NF1 phenotype, but in the deletion of about one-third of the long arm of chromosome 17. This was excluded by molecular analysis of the seven somatic cell hybrids and by cytogenetic analysis [including fluorescence *in situ* hybridization (FISH)] in the remaining cases.

Sequence analysis of deletion junction fragments

The sequence of the amplified 3.4 kb deletion junction fragment from 25 positive *NF1* microdeletion patients was determined by direct cycle sequencing. On the basis of available sequences of NF1REP-P, NF1REP-M and our own sequences of this region, we identified 10 REP-specific nucleotide differences (Fig. 3). Analysis of these nucleotides in the patients revealed that the deletion breakpoints were clustered in a 2 kb region of the junction fragment. Fourteen recombination events occurred in the 670 bp segment, two occurred in a 354 bp segment, and seven in a 967 bp segment (Fig. 3). The parental origin of *de novo* deletions was predominantly maternal, but paternal deletions also occurred at this hotspot (patients 99-2 and 940174). Apparent gene conversion events were detected in two unrelated patients (Fig. 3B). The deletions of *de novo* NF1 patients 984412 and 973287 occurred on their maternally-derived chromosomes. The sequence of their mother's NF1REP-P and -M elements identified polymorphisms that facilitated detection of apparent gene conversion events in the patient's chimeric REP which maximally spanned 907 and 317 bp.

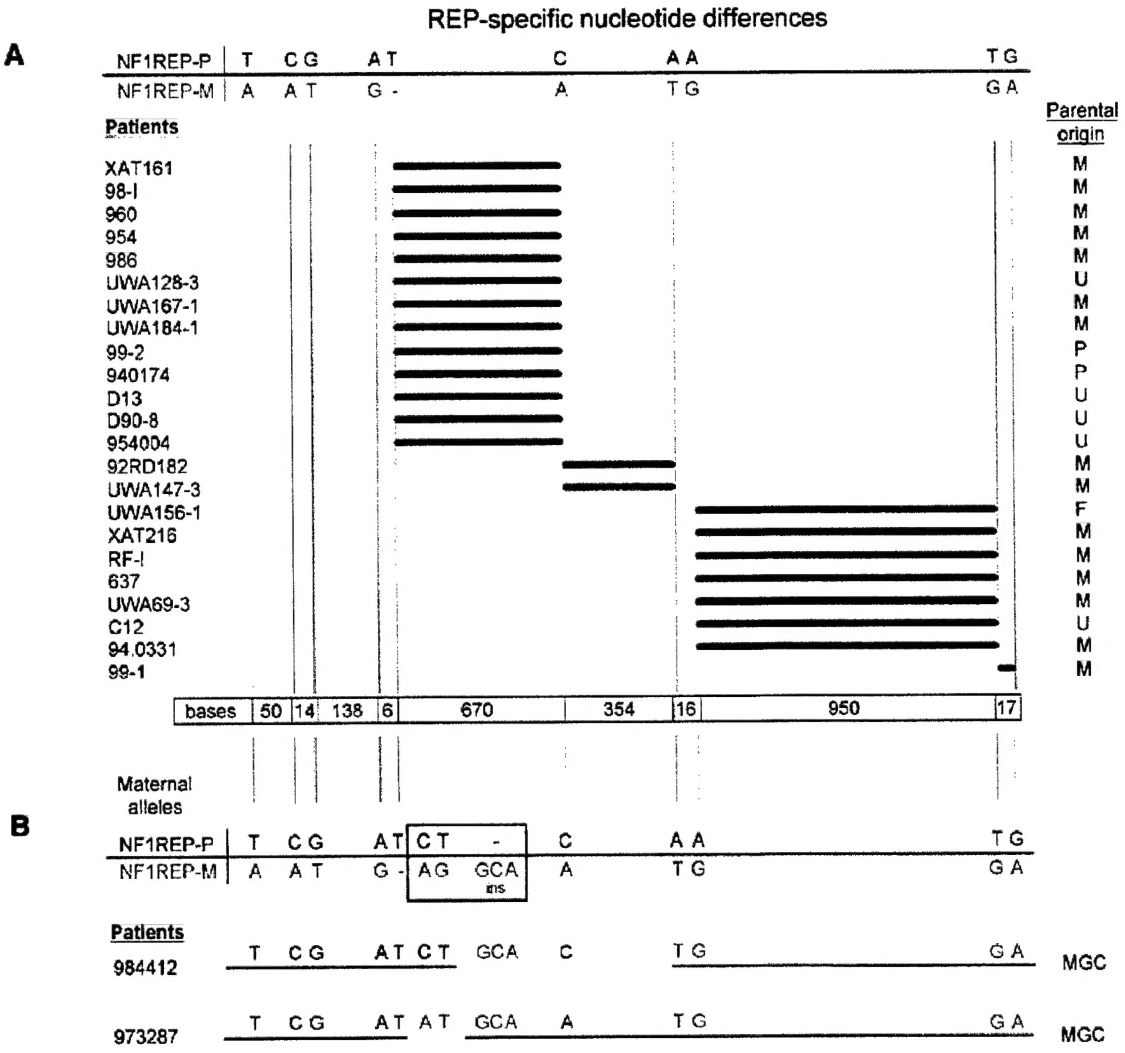

Figure 3. The sites of recombination in 25 *NF1* microdeletion patients. (**A**) REP-P (black) and -M (red) specific nucleotides in the hotspot region are shown at the top. The position of these nucleotides are indicated below based on the sequence of BAC 271K11 (AC005562) from bases 141624 to 144319. For each patient, the black bar delineates the interval in which the recombination event occurred. The parental origin of the *NF1* microdeletion is indicated at the right: M, maternal; P, paternal; F, familial; and U, unknown. (**B**) The deletions in patients 984412 and 973287 were accompanied by apparent maternal gene conversion (MGC). The conversions were detected by comparing the sequence of the patient's deletion junction fragment with that of the corresponding regions of the mother's NF1REP-P (black) and -M (red). Both mothers were heterozygous for additional NF1REP polymorphisms (blue box) which facilitated detection of the conversion events. The gene conversion occurred between positions 142110 and 143017 in patient 984412 and between positions 141993 and 142310 in patient 973287 (numbering based on AC005562).

Sequence analysis and structure of NF1REP-P and -M

To investigate the molecular basis of NF1REP-mediated microdeletion, we examined the nucleotide sequence of the REPs for overall sequence identity, GC content, recombination prone motif, and the existence of potentially disrupted transcripts. The structures of NF1REP-P and -M are such that ~60 kb of the >85 kb paralogs are identical in their arrangement (M. Dorschner and K. Stephens, unpublished data). These 60 kb segments are ~98% identical at the nucleotide level, based on comparisons of the finished sequence of NF1REP-P with the available sequence fragments of the draft sequence of NF1REP-M. Because the sequence quality of NF1REP-M is unknown at this time, the precise degree of nucleotide identity of NF1REP-P and -M may be slightly higher or lower. The nucleotide identity over the entire length of the paralogs appears to be consistent as far as the sequence is available. Pairwise sequence comparisons across the lengths of NF1REP-P and -M did not identify regions with higher or lower nucleotide identities. The average GC content of the NF1REPs is ~50%, while the 2 kb hotspot is ~12% higher.

There is a small region 40–45 kb centromeric to the recombination hotspot with an above average GC content. We have not identified any recombinations in or adjacent to this second region of above average GC content.

A search for recombinogenic motifs and replication-associated sequences in or near the recombination hotspot (Materials and Methods) revealed a χ-like element in the 670 bp recombination interval (Fig. 3; position 142090 of AC005562). WI-12393 is an EST located within each REP (17). A preliminary examination of the gene that includes this EST suggests that it may be disrupted by the deletions described here. It is unknown, however, whether this transcript represents a functional gene or an expressed pseudogene (data not shown).

DISCUSSION

Using an *NF1* microdeletion junction-specific PCR assay, we demonstrated that the recombination event between the NF1REP-P and -M paralogs occurred in a 3.4 kb fragment in 46% of cases with deletions that spanned the *NF1* gene. Sequence and SSCP analyses of the NF1REPs and junction fragments identified REP-specific polymorphisms that enabled us to narrow the breakpoints to a 1889 bp interval near the telomeric end of the NF1REPs (Fig. 1). Within this segment, recombination events clustered in three intervals (Fig. 3). There was no simple correlation between the recombination site and the parental chromosome which underwent deletion. The majority of *de novo* microdeletions occurred preferentially on the maternally-derived homolog, consistent with previous data (6,19). Importantly, however, deletion of paternally-derived chromosomes can also occur in this hotspot region (Fig. 3).

Although a number of deletion/duplication disorders are caused by recombination between flanking paralogs (reviewed in ref. 20), this is only the second to be analyzed at the nucleotide level. The breakpoints have been sequenced for duplications and deletions that cause CMT1A and HNPP diseases, respectively. These two different neuropathies are caused by recombination between flanking CMT1A-REP paralogs. The disease phenotype of CMT1A or HNPP depends upon whether the patient carries a duplication or a deletion of the dosage-sensitive *PMP22* gene located between the CMT1A-REPs (reviewed in refs 21 and 22). There are striking parallels and differences between *NF1* microdeletion and CMT1A/HNPP rearrangements. Each REP-mediated recombination event results in a 1.5 Mb rearrangement, yet the NF1REP is over twice the length of the CMT1A-REP. Both rearrangements show parent-of-origin effects. Eighty percent of *NF1* deletions are maternal in origin and are generated primarily by unequal meiotic crossing over between chromosome 17 homologs (6,18,19). CMT1A duplications are paternal in origin (92%) and also arise by unequal meiotic crossing over between chromosome 17 homologs (23). Maternal rearrangements, albeit CMT1A duplication or HNPP deletion, occur by unequal intrachromatid exchange or excision of an intrachromatidal loop, respectively. Both the 85 kb NF1REPs and the 24 kb CMT1A-REPs have discrete recombination hotspots of 2 kb and 557 bp (24), respectively. χ-like sequences are located in or near both recombination hotspots (Fig. 3) (25). In *Escherichia coli*, χ elements stimulate recombination in their general vicinity but whether they can function in a similar manner in humans remains to be verified by experimental data. In addition, a *mariner*-like transposable element lies ~700 bp from the CMT1A hotspot. This element does not express functional transposase, but it may be a target for a transposase expressed from other such elements in the genome (25). Although recombination hotspots have been identified within each REP element, this does not necessarily imply that these are high frequency meiotic recombination sites in the genome. Recently, a sperm analysis showed that unequal recombination between the CMT1A-REPs occurs at an average rate for the male genome (~1 cM/Mb) (26). It is possible that the special feature of the recombination hotspot region is the combination of a region of high sequence identity and high GC content. However, until now we were unable to find a recombination in the only other region with an above average GC content. Due to the limited availability of sequence from the medial REP (draft quality, unassembled clones) it is impossible at this time to know if other regions of exact sequence identity exist.

Our findings of a recombination hotspot for *NF1* microdeletions and the development of a deletion junction-specific PCR assay have significant implications for research and patient care. *NF1* mutations are typically private and scattered throughout the 8.5 kb coding region, making detection difficult (4). Prior to the findings described here, the most prevalent mutation was R1947X, which occurred in ~1.5% ($n = 255$) of patients and is not associated with any particular phenotype (27). The microdeletion hotspot described here probably accounts for ~5% of *NF1* mutations, based on an estimated microdeletion frequency of 10%. The junction-specific PCR assay will facilitate the identification of the first cohort of NF1 patients with the same mutation. Prospective studies are important to determine whether the deletion is predictive of certain clinical manifestations, such as early age at onset of cutaneous neurofibromas. The majority of *NF1* microdeletion patients in the current study were selected by phenotype. To date, available medical records have confirmed that 10 of the 25 patients with deletions at the hotspot showed an early age at onset of cutaneous neurofibromas (<10 years) or an excessive number of tumors relative to their age. A study to assess the phenotype of the remaining patients is in progress.

Although we anticipate that the deletion junction fragment PCR assay may be clinically useful in some cases of NF1, we consider its implementation at this time to be premature. To date, we have screened 75 healthy individuals with the assay conditions as described. We do not know the frequency of false positives nor how it might be affected by minor alterations in assay conditions. It is possible that this recombination is a low frequency event during mitosis of hematopoietic cells in healthy individuals, which could be detected by our robust and sensitive PCR assay. The probability of detecting such false positives may be higher if the deleted cells have a growth advantage. In addition, this assay cannot differentiate a germline *NF1* microdeletion patient from one with a somatic mosaic microdeletion. There are documented cases of somatic mosaicism for an *NF1* microdeletion, although it is not known whether the recombination events occurred at this hotspot (16,28–30). *A priori*, the germline patient might be expected to have an early onset and a heavy burden of cutaneous neurofibromas, while the somatic might be expected to have a later onset with fewer neurofibromas or other manifestations. In addition, the risk of a mosaic patient having an affected child may be considerably less than that of a germline *NF1* deletion

patient. Application of the PCR junction fragment assay to the healthy parents of eight of the 17 *de novo* microdeletion patients described here was negative. Although none of our *de novo* deletion patients appear to have a mosaic parent, one such case has been described (16).

It is unclear at this time where the breakpoints of the remaining 54% of *NF1* microdeletions occur. Preliminary data suggests that there may be additional recombination hotspots in the NF1REP elements. The development of junction-specific PCR assays for other putative recombination sites will be important for diagnosis, genotype/phenotype analyses, and understanding the molecular basis for recombination-prone sites in the genome.

MATERIALS AND METHODS

Subjects and cell lines

Peripheral blood samples were obtained after informed consent from 54 *NF1* microdeletion patients and their parents, when available. Previous reports document molecular confirmation of deletion in most of the patients (6,9,12,17). In newly ascertained patients, *NF1* microdeletions were confirmed by both analysis of polymorphic markers and FISH, as described previously (12). In all cases the microdeletion was known to extend beyond the borders of the *NF1* gene. In addition, rodent/human somatic hybrid cell lines carrying only the deleted chromosome 17 homolog were constructed from a subset of patients (11,17).

Fine mapping of *NF1* deletion breakpoints

Breakpoints were mapped in somatic cell hybrids by direct sequencing and/or SSCP of amplified products. For SSCP, 15 µl of loading buffer (0.5% dextran blue, 95% formamide) was added to 15 µl of amplified product, heated at 95°C for 3 min and snap cooled on ice for 1 min. Thirty microlitres was electrophoresed through a 0.5× MDE-gel (FMC BioProducts, Rockland, ME) for 10 h at 4°C, 400 V and visualized by fluoro-imager after Sybr Green staining. Forward (F) and reverse (R) primers for SSCP analysis were: stSG31654 F, 5′-TGTGAGG-GGCTCTTTCTATTG-3′ and stSG31654 R, 5′-AGAGTGAT-GTTAGCAGCGCA-3′; stSG40093 F, 5′-TGAAGATGTGGA-CCTGCTGA-3′ and stSG40093 R, 5′-TGTTGCCCAGGCTA-GTTTTC-3′; 60T7 F, 5′-ATCCTCCGCTTTTTCTCCTT-3′ and 60T7 R, 5′-GTTTTAGGGGAGGCCTGTTC-3′ (201 bp); 62T7 F, 5′-TGAGAGGCGGGGTGTATTAG-3′ and 62T7 R, 5′-TCC-TTCTCCAGCCATGTTTC-3′ (187 bp); 58T7 F, 5′-GTATG-GGGAGCTGCTTTTCC-3′ and 58T7 R, 5′-TTCTGTGAGA-CCTGGGAAGG-3′ (217 bp); 5562-142s F, 5′-TACTCACC-CCTAGGCCACAG-3′ and 5562-142s R, 5′-ACACACTCA-GGGACCAACCT-3′ (200 bp); 5562-144s F, 5′-TGGCTCCCT-ACTGTGTTTCC-3′ and 5562-144s R, 5′-TCACACAGC-GACTCCTTCAC-3′ (186 bp); 5562-145s F, 5′-AAATCCCG-GCTTCACAGTTA-3′ and 5562-145s R, 5′-GGCTGGTCTCAA-ACTCTTGG-3′ (197bp); and WI-9461 (http://gdbwww.gdb.org/).

A Southern blot of 10 µg of *Bcl*I-digested DNA, electrophoresed through 0.6% agarose and transferred to Hybond N+ membrane (Amersham, Buckinghamshire, UK), was probed with a 200 bp PCR product from the breakpoint region (5562-142S). The membrane was washed with 2× SSC and 0.1% SDS at 60°C for 30 min, 0.1× SSC and 0.1% SDS at 70°C for 2× 30 min, and exposed to Hyperfilm MP (Amersham) at −70°C for 72 h.

NF1 deletion junction fragment analysis

The 3.4 kb deletion junction fragment was amplified with primers DCF 5′-TCAACCTCCCAGGCTCCCGAA-3′ and DTR 5′-AGCCCCGAGGGAATGAAAAGC-3′. A 25 µl PCR was performed using the Expand Long Template PCR System (Roche Molecular Systems, Indianapolis, IN) with 300 ng DNA, 15 pmoles each primer, 0.35 mM dNTPs, 10× PCR buffer 1, and 2.5 U DNA polymerase. After heating to 94°C for 3 min, samples were subjected to 35 cycles of 94°C for 30 s and 68°C for 2.5 min, with a final extension of 7 min at 68°C. Five microlitres of product was electrophoresed through a 1% agarose gel and visualized by EtBr staining.

Junction fragment products were sequenced by cycle sequencing using either the SequiTherm EXCEL II Long Read (Epicentre, Madison, WI) or the Big-Dye Terminator (Applied Biosystems, Foster City, CA) kits. Extension products were analyzed on either an A.L.F. (Automated Laser Fluorescence sequencer, Pharmacia, Uppsala, Sweden) or an ABI 377 sequencer (Applied Biosystems). Raw nucleotide sequences were analyzed with Sequencher (GeneCodes, Ann Arbor, MI), Clustal W (31), and the vector NTI program (Informax, North Bethesda). The nucleotide sequence of the hotspot region was analyzed for a number of recombination prone motifs including: χ from *E.coli* (5′-GCTGGTGG-3′), yeast Ade6-M26 heptamer (5′-ATGACGT-3′), XY32 homopyrimidine (5′-AAGGGAGAARG-GGTATAGGGRAAGAGGGAA-3′), retroposon LTR (5′-TCA-TACACCACGCAGGGGTAGAGG-3′), LTR-IS (5′-TGGAAA-TCCCC-3′), human minisatellite core sequence (5′-GGGCAG-GAG-3′), two hypervariable minisatellites (5′-GGAGGTGGGC-AGGARG-3′) and (5′-AGAGGTGGGCAGGTGG-3′), translin consensus 1 (5′-GCNC[A/T][G/C][G/C][A/T] $N_{(0-2)}$ GCCC[A/T][G/C] [G/C][A/T]-3′) and consensus 2 (5′-[C/A]TGCAG $N_{(0-4)}$ GCCC[A/T][G/C][G/C][A/T]-3′), and the binding site for the protein *pur* (5′-GGNNGAGGGAGARRRR-3′). In addition, we searched for sequences associated with DNA replication including *Saccharomyces cerevisiae* autonomously replicating sequence (ARS) (5′-WTTTATRTTTW-3′), *Schizosaccharomyces pombe* ARS consensus (5′-WRTTTATTTAW-3′), consensus scaffold attachment regions (5′-AATAAAYAAA-3′, 5′-TTWTWTTWTT-3′, 5′-WADAWAYAWW-3′ and 5′-TWWT-DTTWWW-3′), topoisomerase II binding site (5′-GTNWAYAT-TNATNNR-3′), and human replication origin consensus (5′-WAW-TTDDWWWDHWGWHMAWTT-3′). The recombinogenic and DNA replication-associated motifs were described previously (32 and references therein).

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